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## **B2-kinin receptor plays a key role in B1-, angiotensin converting enzyme inhibitor-, and vascular endothelial growth factor-stimulated in vitro angiogenesis in the hypoxic mouse heart**

Sanchez de Miguel, L ; Neysari, S ; Jakob, S ; Petrimpol, M

**Abstract:** AIMS: Angiotensin converting enzyme (ACE) inhibition reduces heart disease and vascular stiffness in hypertension and leads to kinin accumulation. In this study, we analysed the role and importance of two kinin receptor subtypes in angiogenesis during ACE inhibition in an in vitro model of angiogenesis of the mouse heart. **METHODS AND RESULTS:** First, we analysed the angiogenic properties of bradykinin and enalapril on wild-type C57Bl/6 and B2 receptor(-/-) mouse heart under normoxia (21% O<sub>2</sub>) and hypoxia (1% O<sub>2</sub>) in vitro and the contribution of B1 and B2 kinin receptors to this effect. Bradykinin induced dose-dependent endothelial sprout formation in vitro in adult mouse heart only under hypoxia (1.7 fold, n = 6, P < 0.05). The B2 receptor mediated sprouting that was induced by bradykinin and vascular endothelial growth factor (VEGF(164); n = 6, P < 0.05), but did not mediate sprouting that was induced by growth factors bFGF or PDGF-BB. Enalapril induced sprouting through both the B1 and B2 kinin receptors, but it required the presence of the B2 receptor in both scenarios and was dependent on BK synthesis. B1-receptor agonists induced sprout formation via the B1 receptor (2.5 fold, n = 6, P < 0.05), but it required the presence of the B2 receptor for them to do so. Both B2-receptor and B1-receptor agonist-induced angiogenesis required nitric oxide biosynthesis. **CONCLUSION:** The kinin B2 receptor plays a crucial role in angiogenesis that is induced by different vasoactive molecules, namely bradykinin, ACE inhibitors, B1-stimulating kinin metabolites, and VEGF164 in an in vitro model of angiogenesis of mouse heart under hypoxia. Therapeutic treatment of hypertensive patients by using ACE inhibitors may potentially benefit the ischaemic heart through inducing B2-dependent heart neovascularization.

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# B2-kinin receptor plays a key role in B1-, angiotensin converting enzyme inhibitor-, and vascular endothelial growth factor-stimulated *in vitro* angiogenesis in the hypoxic mouse heart

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Heart;  
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**Aims** Angiotensin converting enzyme (ACE) inhibition reduces heart disease and vascular stiffness in hypertension and leads to kinin accumulation. In this study, we analysed the role and importance of two kinin receptor subtypes in angiogenesis during ACE inhibition in an *in vitro* model of angiogenesis of the mouse heart.

**Methods and results** First, we analysed the angiogenic properties of bradykinin and enalapril on wild-type C57Bl/6 and B2 receptor<sup>-/-</sup> mouse heart under normoxia (21% O<sub>2</sub>) and hypoxia (1% O<sub>2</sub>) *in vitro* and the contribution of B1 and B2 kinin receptors to this effect. Bradykinin induced dose-dependent endothelial sprout formation *in vitro* in adult mouse heart only under hypoxia (1.7 fold,  $n = 6$ ,  $P < 0.05$ ). The B2 receptor mediated sprouting that was induced by bradykinin and vascular endothelial growth factor (VEGF<sub>164</sub>;  $n = 6$ ,  $P < 0.05$ ), but did not mediate sprouting that was induced by growth factors bFGF or PDGF-BB. Enalapril induced sprouting through both the B1 and B2 kinin receptors, but it required the presence of the B2 receptor in both scenarios and was dependent on BK synthesis. B1-receptor agonists induced sprout formation via the B1 receptor (2.5 fold,  $n = 6$ ,  $P < 0.05$ ), but it required the presence of the B2 receptor for them to do so. Both B2-receptor and B1-receptor agonist-induced angiogenesis required nitric oxide biosynthesis.

**Conclusion** The kinin B2 receptor plays a crucial role in angiogenesis that is induced by different vasoactive molecules, namely bradykinin, ACE inhibitors, B1-stimulating kinin metabolites, and VEGF<sub>164</sub> in an *in vitro* model of angiogenesis of mouse heart under hypoxia. Therapeutic treatment of hypertensive patients by using ACE inhibitors may potentially benefit the ischaemic heart through inducing B2-dependent heart neovascularization.

## 1. Introduction

Angiotensin converting enzyme (ACE) inhibitors and bradykinin (BK) have protective effects on ischaemic heart disease and postischaemic brain injury.<sup>1,2</sup> This may be partially due to the reduction of ischaemia as a result of angiogenesis<sup>3</sup> and vasodilation<sup>3,4</sup> during ACE inhibition. ACE inhibitors are known to prevent kinin degradation<sup>5</sup> and some of the properties of ACE inhibition, such as microvascular growth, have been attributed to the accumulation of BK.<sup>6</sup>

Kinins such as BK are released by the kallikrein-kininogen system and induce vasodilation and inflammation and modulate vascular growth. The biological action of kinins is mediated by at least two known G-protein-coupled receptors, the B1 receptors and B2 receptors.<sup>7</sup> BK exerts most of its effects via the B2 receptor that is constitutively expressed in the vasculature. In contrast, the B1 receptor has a higher affinity for kinin metabolites, such as [des-Arg<sup>9</sup>]-BK and [des-Arg<sup>10</sup>]-kallidin, and is expressed during tissue injury or inflammation.<sup>8</sup> Kinin receptor activation signals through NO-cGMP and prostacyclin-AMP pathways, modulating a broad spectrum of cellular functions.<sup>9</sup>

ACE inhibitors have been shown to induce angiogenesis via B2 receptor signalling in hindlimb ischaemia in mice.<sup>10,11</sup>

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In line, BK promotes angiogenesis through B2 receptor signaling by increasing vascular permeability, and vascular endothelial growth factor (VEGF),<sup>12</sup> and B2-receptor antagonism reduces angiogenesis and tumour growth in rats.<sup>13</sup> Our previous studies have demonstrated that angiotensin II (Ang II)-induced angiogenesis *in vitro* is mediated via an AT2-B2 receptor pathway through an Ang II-stimulated BK release, too.<sup>14</sup> However, BK stimulation has also been shown to increase endothelial cell proliferation through the B1 receptor via the nitric oxide (NO) pathway.<sup>15</sup> Similarly, ischaemia has been shown to induce new functional blood vessels through the B1 receptor and to lead to limb post-ischaemic recovery in mice.<sup>16</sup> In the same study, both the B1 receptor and the B2 receptor mediated angiogenesis in response to local delivery of the human-tissue kallikrein gene,<sup>16</sup> similar as in models of rat sponge implants,<sup>17</sup> and in corpora lutea.<sup>18</sup>

Thus, both kinin receptors, either individually or together, have been shown to play a role in angiogenesis during ACE inhibition or the local delivery of kinins.

In the present study, we, therefore, further analyse the role, importance, and crosstalk of the two kinin receptor subtypes in angiogenesis during ACE inhibition by using an *in vitro* model of angiogenesis in mouse heart under hypoxia.<sup>14,19</sup>

## 2. Methods

### 2.1 Animals

Twelve- to 14-week-old C57BL/6 mice (Jackson Laboratories; wild type and B2 receptor<sup>-/-</sup>) were used for angiogenesis *in vitro* assay. The animals were euthanized, the hearts transferred to PBS, and processed within half an hour post mortem as previously described.<sup>19</sup> All experiments conform with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), were in accordance with the Swiss Federal Act on Animal Protection (1998) and were approved by the Veterinary Department of the Kanton of Basel-Stadt (Switzerland).

### 2.2 Angiogenesis *in vitro* assay

We used a technique for three-dimensional *in vitro* angiogenesis assay that we have developed and described in detail in earlier studies.<sup>14,19</sup> In brief, 0.5–1 mm<sup>3</sup> of tissue from the left ventricular myocardium of mouse hearts were embedded into stabilized fibrin-gels (Sigma-Aldrich, Buchs, Switzerland) in 48 well plates. Heart explants were incubated with a stimulant with or without inhibitors under normoxia (21% O<sub>2</sub>) or hypoxia (1% O<sub>2</sub>) for 7 days. Stimulants or inhibitors were added every other day: mouse VEGF<sub>164</sub> (R&D systems); human basic fibroblast growth factor (bFGF)(R&D systems); human platelet-derived growth factor-BB (PDGF-BB) (R&D systems); HOE140 (Sigma-Aldrich AG); BK Acetate, [Lys-des-Arg<sup>9</sup>]-BK, [des-Arg<sup>9</sup>]-BK, [des-Arg<sup>10</sup>]-HOE140, and Lys-(des-Arg<sup>9</sup>, Leu<sup>8</sup>)-BK trifluoroacetate salt (Sigma-Aldrich AG), PKSI-527 (Wako Chemicals), ZM323881 (Calbiochem), GSNO (Sigma-Aldrich), and NO synthase inhibitors (Sigma-Aldrich AG). After 7 days of culture, endothelial sprouts were photographed digitally (ColorView II-Soft Imaging System) on an inverted light microscope (Olympus IX50) and pictures enhanced digitally by ImageJ-software (Wayne Rasband, NIH, MD, USA).

Sprout formation was calculated and averaged by two independent investigators by comparing samples in octuplicates for each condition with a standardized scale (angiogenic index as determined by computer assisted morphometric quantification AnalySIS Pro, Soft Imaging System) as described previously.<sup>19,20</sup> Outgrowing cells and sprouts were characterized by immunostainings with specific endothelial cell marker GSL I-IB<sub>4</sub> (20 µg/mL; Rectolab).<sup>19</sup>

### 2.3 Nitric oxide production assay

NO concentration in the medium from cultured organ explants were measured by using a fluorometric nitrite assay<sup>21</sup> with the NO Assay kit according to the manufacturer's specifications (Calbiochem, Basel, Switzerland). Nitrite accumulated in supernatants was detected by fluorescence in a plate reader (Spectra Max analyzer, Bucher Biotech AG, Basel).

### 2.4 Western blotting analysis

Heart tissue was incubated during 24 and 48 h and lysed in RIPA buffer as described previously.<sup>22</sup> After SDS-PAGE, proteins were transferred onto a polyvinylidene fluoride membrane (Millipore). The membrane was blocked with 4% skim milk powder in a TBS-Tween solution and then probed with polyclonal anti-B1 (M-19, Santa Cruz Biotechnology, CA, USA), monoclonal anti-β-actin (AC-15, Sigma), and finally HRP-conjugated IgGs (Cell Signalling Technology) to visualize proteins bands by chemiluminescence (Amersham).

### 2.5 Statistical analysis

All results represent experiments repeated using heart explants from at least five different mice. Data points in the results represent the mean ± SEM. Statistical analysis was performed with SPSS for Mac OS X (SPSS Inc.). Statistical significance ( $P < 0.05$ ) was computed by using the Kruskal-Wallis and the Mann-Whitney tests for non-parametric analysis and then corrected for multiple comparisons by using the Dunn's test.

## 3. Results

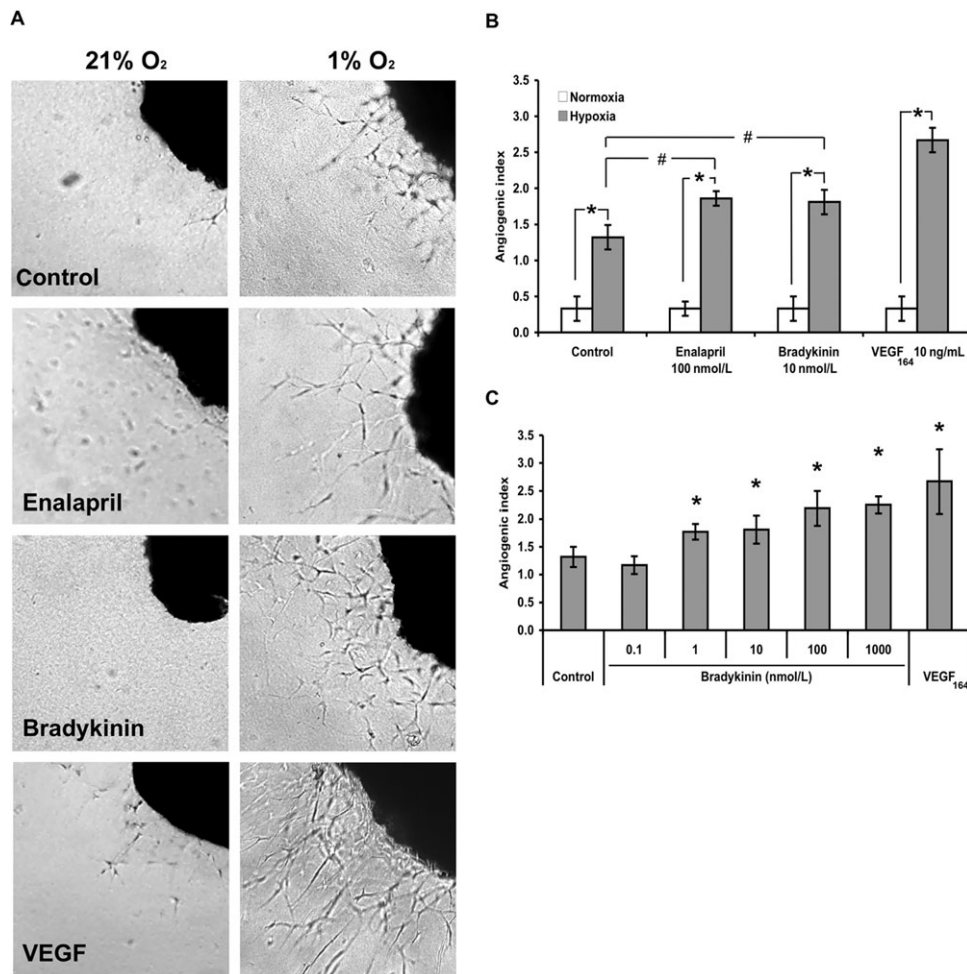
### 3.1 Angiogenic properties of BK and enalapril

Under normoxia, no angiogenic response was elicited. In hypoxia (1% O<sub>2</sub>), however, BK (10<sup>-8</sup> mol/L), enalapril (10<sup>-7</sup> mol/L), and VEGF<sub>164</sub> (10 ng/mL) induced a significant angiogenic response (1.4-, 1.4-, and 2.0-fold, respectively), compared with the negative control  $P < 0.05$  (Figure 1A and B) in heart pieces from wild-type mice. This finding is supported by the results of earlier studies.<sup>14,19,23</sup> Because hypoxia was clearly a pre-requisite for *in vitro* angiogenesis of mouse heart, all of the remaining experiments were performed under hypoxia. The angiogenic index under hypoxia without further stimulation varied between hearts derived from individual mice and ranged from 0.5 to 1.5.

BK-induced endothelial sprouting under hypoxia was dose dependent (10<sup>-9</sup>–10<sup>-6</sup> mol/L BK). A plateau of maximal angiogenic effect of BK was observed at concentrations beyond 10<sup>-6</sup> mol/L (2.25 ± 0.15,  $n = 5$ ,  $P < 0.05$ ) in pieces of heart from wild-type mice (Figure 1C).

### 3.2 Contribution of B1 and B2 receptors in sprout formation

Blocking the B2 receptor completely blunted BK-induced sprout formation ( $P < 0.05$ ) to control levels, whereas blocking the B1 receptor had no effect ( $P = \text{NS}$ ) (Figure 2A). Combining both antagonists did not further reduce BK-induced sprout formation when compared with B2-receptor antagonism (Figure 2A). Accordingly, in heart explants from B2 receptor<sup>-/-</sup> mice, BK did not induce sprouting above control levels at any concentration of BK in a range from 10<sup>-10</sup> to 10<sup>-7</sup> mol/L compared with dose-dependent BK-induced sprouting in explants from wild-type mice (Figure 2B). Thus, BK-induced sprout formation in pieces of mouse heart under hypoxia is specifically mediated through the B2 receptor.



**Figure 1** BK induces sprout formation *in vitro*. (A) Pieces of mouse heart stimulated with BK ( $10^{-8}$  mol/L), enalapril ( $10^{-7}$  nmol/L), mrVEGF<sub>164</sub> (10 ng/mL), or diluent control under normoxia (21% O<sub>2</sub>) or hypoxia (1% O<sub>2</sub>). (B) Quantification of endothelial sprout formation after stimulation with BK ( $10^{-8}$  mol/L), enalapril ( $10^{-7}$  nmol/L), or mrVEGF<sub>164</sub> (10 ng/mL). Angiogenic index is equal to standardized scaled from zero to four that indicates the degree of sprout formation. Data points represent the mean of five independent experiments  $\pm$  SEM. \* $P < 0.05$  vs. normoxia. # $P < 0.05$  vs. control diluent under hypoxia. (C) Quantification of endothelial sprout formation (angiogenic index) after stimulation of mouse heart with increasing concentrations of BK under hypoxia. Data points represent the mean of five independent experiments  $\pm$  SEM. \* $P < 0.05$  vs. negative control.

Interestingly, VEGF did not induce sprouting in B2 receptor<sup>-/-</sup> mouse heart explants either, whereas two other potent angiogenic molecules, bFGF (10 ng/mL) and PDGF-BB (10 ng/mL), induced sprouting in B2 receptor<sup>-/-</sup> hypoxic pieces of mouse heart as strongly as they did in wild-type pieces of mouse heart. Hypoxia-induced angiogenesis in controls was also reduced in B2 receptor<sup>-/-</sup> heart explants when compared with those obtained from hypoxia-induced angiogenesis in wild-type heart explants (Figure 2B).

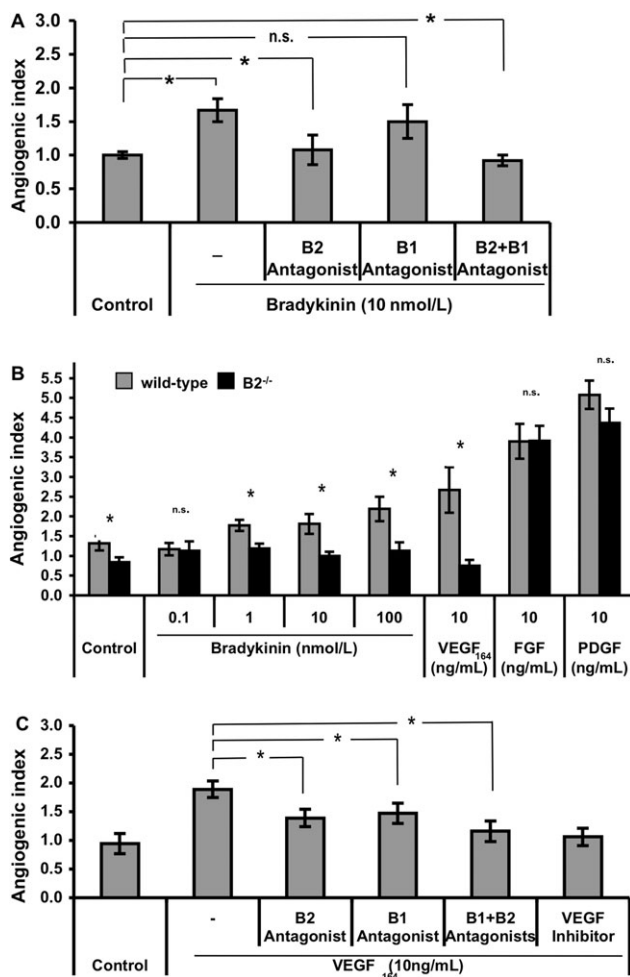
Both B2-receptor and B1-receptor antagonists, alone or in combination reduced VEGF<sub>164</sub>-induced sprout formation in hypoxic heart explants (Figure 2C). VEGF<sub>164</sub> is a ligand for the receptor tyrosine kinase receptors, Flt-1 and Flk-1 responsible for the angiogenic effects of VEGF<sub>164</sub>.<sup>24</sup> The addition of the VEGF receptor kinase inhibitor ZM323881 also blunted VEGF<sub>164</sub>-induced sprout formation. However, bFGF (10 ng/mL)-induced sprout formation was not affected in the presence of the B2-receptor antagonist HOE140 ( $10^{-7}$  mol/L) and the B1-receptor antagonist [des-Arg<sup>10</sup>]-HOE 140 ( $10^{-7}$  mol/L), respectively (data not shown).

Taken together, these results suggest that both BK and VEGF<sub>164</sub> require the B2 receptor for *in vitro* angiogenesis in hypoxic mouse heart explants, but bFGF and PDGF-BB do not.

### 3.3 ACE inhibition effect on sprout formation

We next assessed the angiogenic effects exerted through inhibition of BK degradation by using the two ACE inhibitors enalapril and perindopril. Physiological concentrations of these ACE inhibitors induced sprout formation in mouse heart explants under hypoxia, peaking at  $10^{-7}$  mol/L for enalapril and at  $10^{-5}$  mol/L for perindopril (Figure 3A). Higher and non-physiological concentrations of ACE inhibitors showed lesser angiogenic effects that may be related to the anti-angiogenic effects of metalloproteinase activity inhibition.<sup>25</sup> Pre-incubation of the heart explants with the specific kallikrein inhibitor PKSI-527 ( $10^{-5}$  mol/L) completely inhibited enalapril-induced angiogenesis (Figure 3B). Since inhibition of kallikrein blocks the conversion of kininogens into kinins, latter result suggests that ACE-induced angiogenesis requires kinin-induced signalling. HOE140 ( $10^{-7}$  mol/L), the selective B2-receptor antagonist, reduced enalapril-induced sprout formation significantly. The selective B1-receptor antagonist, [des-Arg<sup>10</sup>]-HOE140<sup>26</sup> ( $10^{-7}$  mol/L), inhibited sprout formation to a similar extent (Figure 3C). The combination of both antagonists did not further inhibit enalapril-induced sprout formation (Figure 3C). The same results were obtained





**Figure 2** BK- and VEGF-induced sprout formation *in vitro* under hypoxia. (A) Quantification of endothelial sprout formation (angiogenic index) of pieces of mouse heart was incubated with BK alone or in the presence of a selective B2-receptor antagonist HOE-140 ( $10^{-7}$  mol/L, B2 Ant) or a selective B1-receptor antagonist [des-Arg<sup>10</sup>]-HOE140 ( $10^{-7}$  mol/L, B1 Ant), or both, under hypoxia. Data points represent the mean of five independent experiments  $\pm$  SEM. \* $P < 0.05$  vs. BK-stimulated heart under hypoxia; n.s. = not significant. (B) Quantification of endothelial sprout formation (angiogenic index) of wild-type or B2 receptor<sup>-/-</sup> mouse heart explants stimulated with increasing concentrations of BK, mrVEGF<sub>164</sub>, bFGF, or PDGF-BB under hypoxia (1% O<sub>2</sub>). Data points represent the mean of six independent experiments  $\pm$  SEM. \* $P < 0.05$  vs. wild-type heart. (C) Quantification of endothelial sprout formation (angiogenic index) of mouse heart explants incubated with mrVEGF<sub>164</sub> alone or in the presence of the selective B2-receptor antagonist HOE-140 ( $10^{-7}$  mol/L, B2 Ant), the selective B1-receptor antagonist [des-Arg<sup>10</sup>]-HOE140 ( $10^{-7}$  mol/L, B1 Ant), both B1- and B2-receptor antagonists or VEGF inhibitor ZM323881 ( $10^{-7}$  mol/L, VEGF Inh) under hypoxia. Data points represent the mean of five independent experiments  $\pm$  SEM. \* $P < 0.05$  vs. VEGF<sub>164</sub>-stimulated angiogenesis under hypoxia.

when explants were stimulated with perindopril  $10^{-6}$  mol/L (data not shown). Like BK, ACE inhibitors did not induce sprout formation in pieces of B2 receptor<sup>-/-</sup> mouse heart (Figure 3D). These results indicate that angiogenesis induced by ACE inhibitors under hypoxia is kinin-induced and can be driven through either the B1 or B2 receptor, but requires the presence of the B2 receptor for either scenario.

### 3.4 Activation of B1 receptors with B1 receptor agonists

To assess the role of B1 receptor activation, we used selective B1 receptor agonists.<sup>9</sup> Both of the selective B1-receptor

agonists, ([des-Arg<sup>9</sup>]-BK and [des-Arg<sup>10</sup>]-kallidin, induced sprout formation in the mouse heart explants under hypoxia (2.5-fold increase with each B1 agonist at a concentration of  $10^{-7}$  mol/L,  $P < 0.05$ ) (Figure 4A). As expected, incubation with the selective B1-receptor antagonist [des-Arg<sup>10</sup>]-HOE140 blocked the angiogenic effect of the B1-receptor agonist ([des-Arg<sup>9</sup>]-BK,  $10^{-7}$  mol/L), whereas the B2-receptor antagonist HOE-140 had no effect (Figure 4B). The same results were observed when we used the B1-receptor agonist [des-Arg<sup>10</sup>]-kallidin (data not shown). However, B1-receptor agonists did not induce any sprout formation in pieces of B2 receptor<sup>-/-</sup> mouse heart under hypoxia (Figure 4C) suggesting that B1 receptor-induced angiogenesis requires the presence of the B2 receptor.

Since B1-receptor expression is not constitutive but inducible, we assessed the presence of B1-receptor protein in the hypoxic mouse heart. B1 receptor protein was present in both wild-type and B2-receptor<sup>-/-</sup> mouse heart under hypoxia, and its expression appeared to diminish after the first 24 h of incubation under normoxia (Figure 4D).

### 3.5 BK-induced sprouts resemble VEGF- but not FGF-induced sprouting

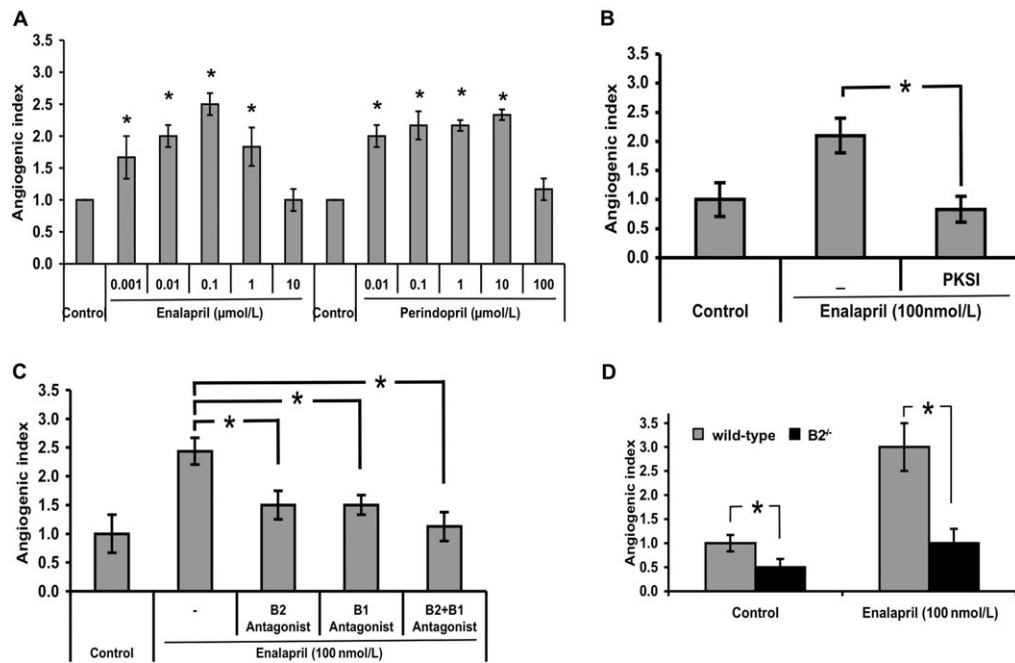
We characterized sprout formation under hypoxia by analysing the morphology and cell type. All tested vasoactive peptides (BK, BK1-agonist), ACEI (enalapril), and angiogenic factors (VEGF, bFGF, and PDGF-BB) induced organized sprouts and tubes (Figure 5A). Specific endothelial marker, Alexa Fluor labelled isolectin (GSL I-IB<sub>4</sub>), shows that these sprouts are composed of endothelial cells (representative example shown for B1 receptor-agonist and bFGF in Figure 5B).

BK-, B1 agonist-, and ACE inhibitor-induced sprouts were more extended and show a low level of complexity and branching (Figure 5A and B). Morphologically these sprouts resemble those induced by VEGF<sub>164</sub> (Figure 5A). On the other side, bFGF- or PDGF-induced sprouting showed a higher level of complexity, with thicker endothelial sprouts and a higher level of network formation and branching. Additionally unorganized single endothelial cells migrated into the fibrin matrix (Figure 5A and B).

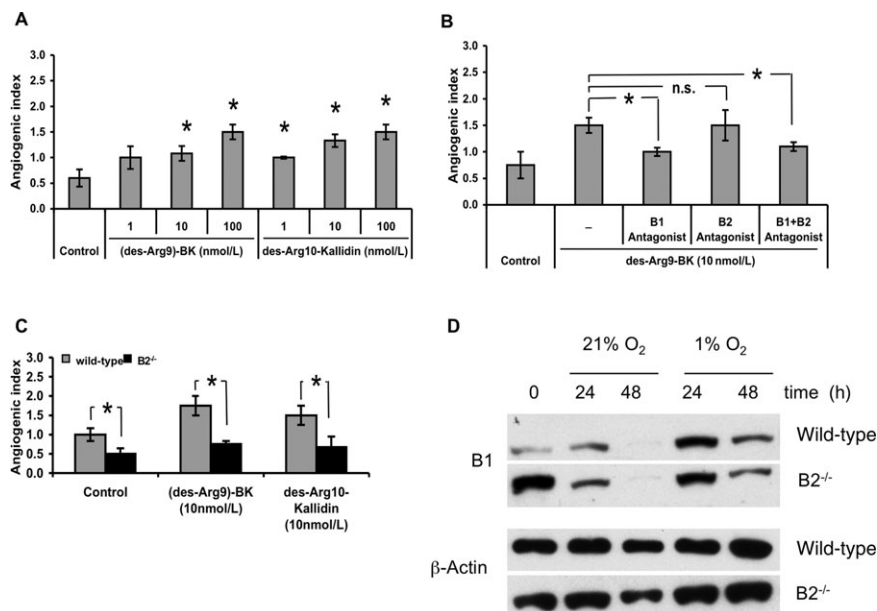
### 3.6 Nitric oxide biosynthesis is required for B1 and B2 receptor-induced angiogenesis

Hypoxia significantly induced NO biosynthesis as measured by nitrite accumulation. BK ( $10^{-7}$  mol/L) further stimulated NO production under hypoxia. B2-receptor antagonist HOE140 ( $10^{-6}$  mol/L) blocked the increase in NO biosynthesis (Figure 6A and B). B1-receptor antagonists but not B2-receptor antagonists inhibited an increase in NO by the B1-receptor agonist [des-Arg<sup>9</sup>]-BK ( $10^{-7}$  mol/L) (Figure 6B).

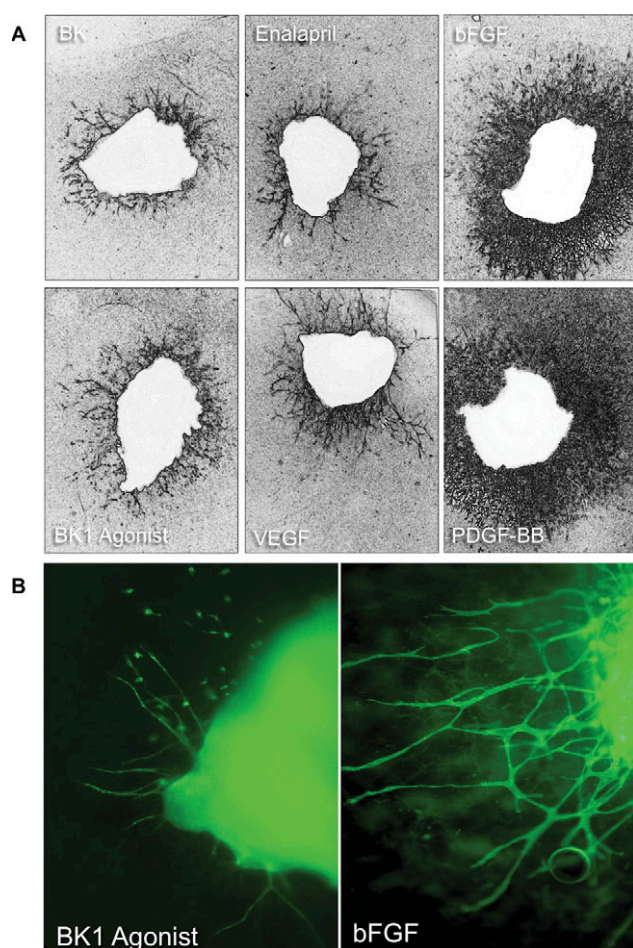
NO synthase inhibitors were added prior to stimulation of the heart pieces with bradykinin ( $10^{-8}$  mol/L) or [des-Arg<sup>9</sup>]-BK ( $10^{-8}$  mol/L). All of the NO synthase inhibitors tested, L-NAME ( $10^{-7}$  mol/L), L-NIO ( $10^{-6}$  mol/L), L-NIL ( $10^{-7}$  mol/L), 1400W ( $10^{-7}$  mol/L), and SMT ( $10^{-7}$  mol/L), prevented sprout formation by both BK and B1 selective receptor agonists (Figure 6C and D). Incubation in the presence of L-NIL ( $10^{-7}$  mol/L) prevented ACE inhibitor-induced sprout formation completely (data not shown). This effect was specific of NO inhibition since addition of an exogenous NO donor, S-nitrosoglutathione, GSNO ( $10^{-5}$  mol/L) reverted the anti-angiogenic effect of L-NAME (Figure 6E). Thus, kinin-induced



**Figure 3** ACE inhibitor-induced sprout formation *in vitro* under hypoxia. (A) Quantification of endothelial sprout formation (angiogenic index) of wild-type mouse heart explants stimulated with increasing concentrations of an ACE inhibitor, either enalapril or perindopril under hypoxia. Data points represent the mean of three independent experiments  $\pm$  SEM.  $^*P < 0.05$  vs. control diluent. (B) Quantification of endothelial sprout formation (angiogenic index) of wild-type mouse explants stimulated with enalapril alone or in the presence of PKSI-527 ( $10^{-5}$   $\mu\text{mol/L}$ ), a specific kininogenase inhibitor, under hypoxia. Data points represent the mean of three independent experiments  $\pm$  SEM.  $^*P < 0.05$  vs. enalapril-induced angiogenesis under hypoxia. (C) Quantification of endothelial sprout formation (angiogenic index) of mouse heart explants incubated with enalapril alone or in the presence of either the selective B2-receptor antagonist HOE-140 ( $10^{-7}$  mol/L) or the selective B1-receptor antagonist [des-Arg<sup>10</sup>]-HOE 140 ( $10^{-7}$  mol/L) or both, under hypoxia. Data points represent the mean of three independent experiments  $\pm$  SEM.  $^*P < 0.05$  vs. enalapril-induced angiogenesis under hypoxia. (D) Quantification of endothelial sprout formation (angiogenic index) wild-type and B2 receptor <sup>-/-</sup> mouse heart explants stimulated with enalapril under hypoxia (1% O<sub>2</sub>). Data points represent the mean of six independent experiments  $\pm$  SEM.  $^*P < 0.05$  vs. wild-type heart.



**Figure 4** B1 receptor agonist-induced sprout formation *in vitro* under hypoxia. (A) Quantification of endothelial sprout formation (angiogenic index) of mouse heart explants stimulated with increasing concentrations of the B1 agonists [des-Arg<sup>9</sup>]-BK or [des-Arg<sup>10</sup>]-kallidin under hypoxia over a period of 7 days. Data points represent the mean of six independent experiments  $\pm$  SEM.  $^*P < 0.05$  vs. control diluent. (B) Quantification of endothelial sprout formation (angiogenic index) of mouse heart explants incubated with [des-Arg<sup>9</sup>]-BK alone or in the presence of either the selective B1-receptor antagonist [des-Arg<sup>10</sup>]-HOE 140 ( $10^{-7}$  mol/L) or the selective B2-receptor antagonist HOE140 ( $10^{-6}$  mol/L) or both, under hypoxia over a period of 7 days. Data points represent the mean of five independent experiments  $\pm$  SEM.  $^*P < 0.05$  vs. [des-Arg<sup>9</sup>]-BK-induced angiogenesis; n.s. = not significant. (C) Quantification of endothelial sprout formation (angiogenic index) of wild-type and B2 receptor <sup>-/-</sup> mouse heart explants stimulated with the B1-receptor agonists: [des-Arg<sup>9</sup>]-BK ( $10^{-8}$  mol/L) or [des-Arg<sup>10</sup>]-kallidin ( $10^{-8}$  mol/L) under hypoxia over a period of 7 days. Data points represent the mean of six independent experiments  $\pm$  SEM.  $^*P < 0.05$  vs. wild-type heart. (D) Quantification of endothelial sprout formation (angiogenic index) of wild-type mice and B2 receptor <sup>-/-</sup> mouse heart explants incubated under 1% O<sub>2</sub> or 21% O<sub>2</sub> during 0, 24, and 48 h. B1 receptor expression was analysed by western blotting.  $\beta$ -actin expression is shown as the control.



**Figure 5** Morphology of the sprouts derived from pieces of mouse heart under hypoxia. (A) Different angiogenic stimuli induce sprouts with different morphology in pieces of mouse heart under hypoxia. BK- (10 nmol/L), des-Arg<sup>9</sup>-BK- (10 nmol/L), enalapril- (100 nmol/L), VEGF<sub>164</sub>- (10 ng/mL), bFGF- (10 ng/mL), and PDGF-BB- (10 ng/mL) induced sprouting was observed under an inverted light microscope. Magnification 4×. (B) Fixed des-Arg<sup>9</sup>-BK- and bFGF-induced sprouts stained for endothelial cells by fluorescein-conjugated GSL-IB4 (green). Magnification 10×.

angiogenesis *in vitro* of heart explants depends on B1- or B2-induced NO biosynthesis.

#### 4. Discussion

In this study, we show that exogenous BK and inhibition of its degradation by ACE inhibitors induce sprout formation under hypoxia in a mouse heart organ culture model. By using selective B1 and B2 antagonists and heart tissue from B2<sup>-/-</sup> mice, we demonstrate that ACE inhibition-induced sprout formation *in vitro* is mediated through the B1 and B2 receptors. ACE inhibitor-induced angiogenesis has been attributed to the B2 receptor in hypertensive rats<sup>27</sup> and in rabbit hindlimb ischaemia,<sup>10</sup> but also to the B1 receptor in coronary endothelium.<sup>28</sup> ACE inhibitors have also been shown to directly bind and activate the B1 receptor in the absence of ACE.<sup>29</sup> In our study, enzymatic activity of kallikrein, which liberates BK and kallidins from kininogens, was additionally required for ACE inhibitor-induced angiogenesis. Further, the physical presence of the B2 receptor was required since no sprouts formed from B2 receptor<sup>-/-</sup> mouse heart explants during ACE inhibitor and B1-agonist stimulation. No biochemical evidence of a direct

binding of ACE inhibitors to the B2 receptor exists so far.<sup>30</sup> Thus, we suggest that ACE-inhibitor-mediated kallidin and BK accumulation activate B1 and B2 receptors, respectively, and that the B2 receptor is required mandatory to induce angiogenesis.

A possible explanation for this effect is the formation of complexes between B1 and B2 receptors permitting the transduction of the B1 receptor signal. Indeed, G-protein coupled receptors can form complexes, i.e. AT2-B2<sup>-</sup>,<sup>31</sup> AT2-AT1 receptors,<sup>32</sup> and also B1-B2.<sup>33</sup> It has been demonstrated that B1-B2 complex formation correlates with increased constitutive and agonist-stimulated receptor signalling efficacy.<sup>33</sup> In these *in vitro* studies, the B2-receptor antagonist HOE-140 did not modify heterocomplex formation, suggesting that B1 agonist signals through the B1-B2 complex even in the presence of B2 antagonists.<sup>33</sup> In our B2<sup>-/-</sup> mouse heart explants, evidently, heterocomplexes cannot form thus preventing signalling through B1 receptor. However, technical limitations of our model do not allow to demonstrate heterodimerization of these receptors.

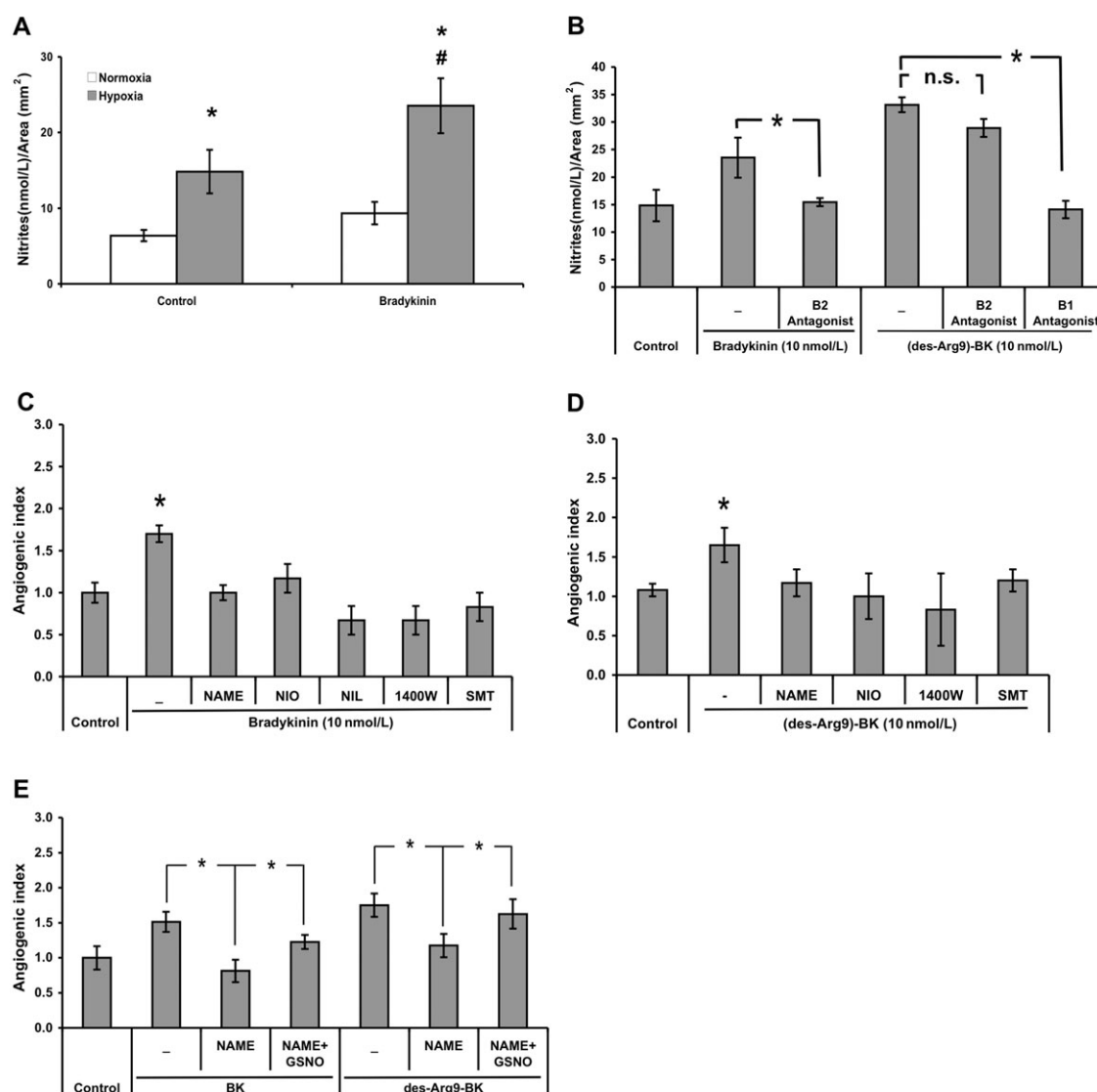
The role of B2 receptor in angiogenesis is not restricted to kinin activation. Also, *in vitro* angiogenesis triggered by the classic angiogenic molecule VEGF was absent in the B2 receptor<sup>-/-</sup> mouse heart explants. This effect was specific for VEGF-induced angiogenesis, since B2 receptor knockout did not reduce bFGF- and PDGF-BB-mediated sprout formation. The morphology of BK-induced and ACE inhibitor-induced endothelial sprouts resembled the morphology of VEGF-induced sprouts but not the morphology of FGF- or PDGF-BB-induced sprouts. A mechanism that may explain this observation is transactivation of the KDR/Flk-1 receptor by the B2 receptor, which leads to endothelial tube formation<sup>34</sup> and contributes to endothelial NO synthase activation<sup>35</sup> in coronary endothelial cells.

We also found that both activation of the B1 receptor and the B2 receptor induced endothelial sprout formation through a mechanism that requires NO biosynthesis. The pro-angiogenic effects of ACE inhibitors have been associated with an increase in NO synthase,<sup>10</sup> and NO biosynthesis is a pre-requisite for angiogenesis.<sup>14,36,37</sup> Other mechanisms, e.g. antioxidant or anti-inflammatory effects of ACE inhibitors may, however, also contribute to angiogenesis by preserving NO.<sup>38</sup>

A physiological role of the B2 receptor in angiogenesis of the heart has already been suggested, as myocardial capillaries display rarefaction of up to 38% in B2 receptor<sup>-/-</sup> mice.<sup>39</sup> These structural changes can affect cardiac remodelling and induce hypertension and cardiac hypertrophy.<sup>39,40</sup> Moreover, B2 receptor<sup>-/-</sup> mice have shown a diminished cardioprotective response to ACE inhibitors.<sup>41</sup> Our experimental model underlines the importance of the kinin receptors in angiogenesis, identifying the B2 receptor as a key receptor to vessel growth that affects not only kinin signalling but also VEGF-induced angiogenesis. The exact nature of the VEGF-B2 interaction as well as the B2-B1 receptor interaction still needs to be clarified.

Thus, treatment of hypertensive patients with ACE inhibitors may improve ischaemic heart function through inducing B2-dependent heart neovascularization. Pathological situations with a dysfunctional B2 receptor expression may not receive adequate benefit from this treatment.<sup>42</sup> In conclusion, our experiments show that angiogenic effects of ACE inhibition in the mouse heart *in vitro* require hypoxia and the presence of both the B1 receptor and the B2





**Figure 6** NO synthesis and BK-induced and B1-receptor agonist-induced sprout formation. (A) Pieces of wild-type mouse heart stimulated with BK under normoxia or hypoxia. Data points represent the mean of three independent experiments  $\pm$  SEM. \* $P < 0.05$  vs. control diluent. <sup>#</sup> $P < 0.05$  vs. normoxia. (B) Pieces of wild-type mouse heart stimulated with BK and [des-Arg<sup>9</sup>]-BK alone or in the presence of the selective B1 antagonist [des-Arg<sup>10</sup>]-HOE140 ( $10^{-7}$  mol/L) or the selective B2 inhibitor HOE140 ( $10^{-7}$  mol/L) under hypoxia. Data points represent the mean of three independent experiments  $\pm$  SEM. \* $P < 0.05$  vs. stimulus under hypoxia; n.s. = not significant. (C) Pieces of type mouse heart stimulated with BK ( $10^{-8}$  mol/L) alone or in the presence of different NO synthase inhibitors ( $10^{-7}$  mol/L) and incubated under hypoxia. Data points represent the mean of five independent experiments  $\pm$  SEM. \* $P < 0.05$  vs. control diluent. (D) Pieces of wild-type mouse heart stimulated with [des-Arg<sup>9</sup>]-BK ( $10^{-8}$  mol/L) alone or in the presence of different NO synthase inhibitors ( $10^{-7}$  mol/L) and incubated under hypoxia. Data points represent the mean of five independent experiments  $\pm$  SEM. \* $P < 0.05$  vs. control diluent. (E) Pieces of wild-type mouse heart stimulated with BK ( $10^{-8}$  mol/L) or [des-Arg<sup>9</sup>]-BK ( $10^{-8}$  mol/L) alone or in the presence of the NO synthase inhibitor, L-NAME ( $10^{-7}$  mol/L) with or without the exogenous NO donor, S-nitrosoglutathione (GSNO) ( $10^{-5}$  mol/L) and incubated under hypoxia. Data points represent the mean of three independent experiments  $\pm$  SEM. \* $P < 0.05$  vs. stimulus incubated with L-NAME.

receptor with NO as a downstream effector. The presence of the B2 receptor is essential for angiogenesis that is induced by hypoxia, BK, kinin metabolites, ACE inhibition, and VEGF. Further studies may reveal the nature of the interactions between kinin receptors and its relevance to hypoxia-induced and VEGF-induced angiogenesis in physiological and pathological responses.

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## References

- Li K, Chen X. Protective effects of captopril and enalapril on myocardial ischemia and reperfusion damage of rat. *J Mol Cell Cardiol* 1987;19: 909–915.



2. Xia CF, Smith RS Jr, Shen B, Yang ZR, Borlongan CV, Chao L *et al.* Postischemic brain injury is exacerbated in mice lacking the kinin B2 receptor. *Hypertension* 2006;**47**:752–761.
3. Fabre JE, Rivard A, Magner M, Silver M, Isner JM. Tissue inhibition of angiotensin-converting enzyme activity stimulates angiogenesis in vivo. *Circulation* 1999;**99**:3043–3049.
4. Tanonaka K, Kamiyama T, Takezono A, Sakai K, Takeo S. Beneficial effects of angiotensin I converting enzyme inhibitor on post-ischemic contractile function of perfused rat heart. *J Mol Cell Cardiol* 1996;**28**:1659–1670.
5. Yang HY, Erdos EG, Levin Y. A dipeptidyl carboxypeptidase that converts angiotensin I and inactivates bradykinin. *Biochim Biophys Acta* 1970;**214**:374–376.
6. Olivetti G, Cigola E, Lagrasta C, Ricci R, Quaini F, Monopoli A *et al.* Spirapril prevents left ventricular hypertrophy, decreases myocardial damage and promotes angiogenesis in spontaneously hypertensive rats. *J Cardiovasc Pharmacol* 1993;**21**:362–370.
7. Allogho SN, Gobeil F, Pheng LH, Nguyen-Le XK, Neugebauer W, Regoli D. Kinin B1 and B2 receptors in the mouse. *Can J Physiol Pharmacol* 1995;**73**:1759–1764.
8. Couture R, Harrisson M, Vianna RM, Cloutier F. Kinin receptors in pain and inflammation. *Eur J Pharmacol* 2001;**429**:161–176.
9. Leeb-Lundberg LM, Marceau F, Muller-Esterl W, Pettibone DJ, Zuraw BL. International union of pharmacology. XLV. Classification of the kinin receptor family: from molecular mechanisms to pathophysiological consequences. *Pharmacol Rev* 2005;**57**:27–77.
10. Silvestre JS, Bergaya S, Tamarat R, Duriez M, Boulanger CM, Levy BI. Proangiogenic effect of angiotensin-converting enzyme inhibition is mediated by the bradykinin B(2) receptor pathway. *Circ Res* 2001;**89**:678–683.
11. Ebrahimi TG, Tamarat R, Clergue M, Duriez M, Levy BI, Silvestre JS. Dual effect of angiotensin-converting enzyme inhibition on angiogenesis in type 1 diabetic mice. *Arterioscler Thromb Vasc Biol* 2005;**25**:65–70.
12. Ishihara K, Kamata M, Hayashi I, Yamashina S, Majima M. Roles of bradykinin in vascular permeability and angiogenesis in solid tumor. *Int Immunopharmacol* 2002;**2**:499–509.
13. Ikeda Y, Hayashi I, Kamoshita E, Yamazaki A, Endo H, Ishihara K *et al.* Host stromal bradykinin B2 receptor signaling facilitates tumor-associated angiogenesis and tumor growth. *Cancer Res* 2004;**64**:5178–5185.
14. Munk VC, de Miguel LS, Petrimpol M, Butz N, Banfi A, Eriksson U *et al.* Angiotensin II induces angiogenesis in the hypoxic adult mouse heart in vitro through an AT2-B2 receptor pathway. *Hypertension* 2007;**49**:1178–1185.
15. Parenti A, Morbidelli L, Ledda F, Granger HJ, Ziche M. The bradykinin/B1 receptor promotes angiogenesis by up-regulation of endogenous FGF-2 in endothelium via the nitric oxide synthase pathway. *FASEB J* 2001;**15**:1487–1489.
16. Emanuelli C, Minasi A, Zacheo A, Chao J, Chao L, Salis MB *et al.* Local delivery of human tissue kallikrein gene accelerates spontaneous angiogenesis in mouse model of hindlimb ischemia. *Circulation* 2001;**103**:125–132.
17. Hayashi I, Amano H, Yoshida S, Kamata K, Kamata M, Inukai M *et al.* Suppressed angiogenesis in kininogen-deficiencies. *Lab Invest* 2002;**82**:871–880.
18. Plendl J, Snyman C, Naidoo S, Sawant S, Mahabeer R, Bhoola KD. Expression of tissue kallikrein and kinin receptors in angiogenic microvascular endothelial cells. *Biol Chem* 2000;**381**:1103–1115.
19. Kiefer FN, Munk VC, Humar R, Dieterle T, Landmann L, Battegay EJ. A versatile in vitro assay for investigating angiogenesis of the heart. *Exp Cell Res* 2004;**300**:272–282.
20. Munk VC, Sanchez de Miguel L, Humar R, Kiefer FN, Butz N, Battegay EJ. iNOS is required for in vitro angiogenesis of hypoxic mouse hearts. *Semin Cardiol* 2006;**12**:21–26.
21. Misko TP, Schilling RJ, Salvemini D, Moore WM, Currie MG. A fluorometric assay for the measurement of nitrite in biological samples. *Anal Biochem* 1993;**214**:11–16.
22. Li W, Petrimpol M, Molle KD, Hall MN, Battegay EJ, Humar R. Hypoxia-induced endothelial proliferation requires both mTORC1 and mTORC2. *Circ Res* 2007;**100**:79–87.
23. Banai S, Shweiki D, Pinson A, Chandra M, Lazarovici G, Keshet E. Upregulation of vascular endothelial growth factor expression induced by myocardial ischaemia: implications for coronary angiogenesis. *Cardiovasc Res* 1994;**28**:1176–1179.
24. Millauer B, Witzmann-Voos S, Schnurch H, Martinez R, Moller NP, Risau W *et al.* High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. *Cell* 1993;**72**:835–846.
25. Lods N, Ferrari P, Frey FJ, Kappeler A, Berthier C, Vogt B *et al.* Angiotensin-converting enzyme inhibition but not angiotensin II receptor blockade regulates matrix metalloproteinase activity in patients with glomerulonephritis. *J Am Soc Nephrol* 2003;**14**:2861–2872.
26. Wirth KJ, Wiemer G, Scholkens BA. DesArg10[Hoe 140] is a potent B1 bradykinin antagonist. *Agents Actions Suppl* 1992;**38**:406–413.
27. Gohlke P, Kuwer I, Schnell A, Amann K, Mall G, Unger T. Blockade of bradykinin B2 receptors prevents the increase in capillary density induced by chronic angiotensin-converting enzyme inhibitor treatment in stroke-prone spontaneously hypertensive rats. *Hypertension* 1997;**29**:478–482.
28. Donnini S, Solito R, Giachetti A, Granger HJ, Ziche M, Morbidelli L. Fibroblast growth factor-2 mediates angiotensin-converting enzyme inhibitor-induced angiogenesis in coronary endothelium. *J Pharmacol Exp Ther* 2006;**319**:515–522.
29. Ignjatovic T, Tan F, Brovkovich V, Skidgel RA, Erdos EG. Novel mode of action of angiotensin I converting enzyme inhibitors: direct activation of bradykinin B1 receptor. *J Biol Chem* 2002;**277**:16847–16852.
30. Benzing T, Fleming I, Blaukat A, Muller-Esterl W, Busse R. Angiotensin-converting enzyme inhibitor ramiprilat interferes with the sequestration of the B2 kinin receptor within the plasma membrane of native endothelial cells. *Circulation* 1999;**99**:2034–2040.
31. Abadir PM, Periasamy A, Carey RM, Siragy HM. Angiotensin II type 2 receptor-bradykinin B2 receptor functional heterodimerization. *Hypertension* 2006;**48**:316–322.
32. Abdalla S, Lother H, Quatterer U. AT1-receptor heterodimers show enhanced G-protein activation and altered receptor sequestration. *Nature* 2000;**407**:94–98.
33. Kang DS, Ryberg K, Morgelin M, Leeb-Lundberg LM. Spontaneous formation of a proteolytic B1 and B2 bradykinin receptor complex with enhanced signaling capacity. *J Biol Chem* 2004;**279**:22102–22107.
34. Miura S, Matsuo Y, Saku K. Transactivation of KDR/Flk-1 by the B2 receptor induces tube formation in human coronary endothelial cells. *Hypertension* 2003;**41**:1118–1123.
35. Thuringer D, Maulon L, Frelin C. Rapid transactivation of the vascular endothelial growth factor receptor KDR/Flk-1 by the bradykinin B2 receptor contributes to endothelial nitric-oxide synthase activation in cardiac capillary endothelial cells. *J Biol Chem* 2002;**277**:2028–2032.
36. Kondo T, Kobayashi K, Murohara T. Nitric oxide signaling during myocardial angiogenesis. *Mol Cell Biochem* 2004;**264**:25–34.
37. Kiefer FN, Misteli H, Kalak N, Tschudin K, Fingerle J, Van der Kooij M *et al.* Inhibition of NO biosynthesis, but not elevated blood pressure, reduces angiogenesis in rat models of secondary hypertension. *Blood Press* 2002;**11**:116–124.
38. Steinhilber S, Pehlivanli S, Bakovic-Alt R, Meiser BM, Becker BF, von Scheidt W *et al.* Beneficial effects of quinaprilat on coronary vasomotor function, endothelial oxidative stress, and endothelin activation after human heart transplantation. *Transplantation* 2004;**77**:1859–1865.
39. Maestri R, Milia AF, Salis MB, Graiani G, Lagrasta C, Monica M *et al.* Cardiac hypertrophy and microvascular deficit in kinin B2 receptor knockout mice. *Hypertension* 2003;**41**:1151–1155.
40. Madeddu P, Varoni MV, Palomba D, Emanuelli C, Demontis MP, Glorioso N *et al.* Cardiovascular phenotype of a mouse strain with disruption of bradykinin B2-receptor gene. *Circulation* 1997;**96**:3570–3578.
41. Yang XP, Liu YH, Mehta D, Cavaasin MA, Shesely E, Xu J *et al.* Diminished cardioprotective response to inhibition of angiotensin-converting enzyme and angiotensin II type 1 receptor in B(2) kinin receptor gene knockout mice. *Circ Res* 2001;**88**:1072–1079.
42. Van Guilder GP, Pretorius M, Byrd JB, Hill K, Gainer JV *et al.* Bradykinin type 2 receptor BE1 genotype influences bradykinin-dependent vasodilation during angiotensin-converting enzyme inhibition. *Hypertension* 2008;**51**:454–459.